and apoptosis in these cells. Time-lapse videos showed neighboring cells also underwent apoptosis, but expression of Bax and/or Bak was essential to this effect as no bystanders were observed in cells lacking both of these MAC components. In osteosarcoma cell lines, this effect relied upon gap junction intercellular communication, as bystander cell death was abrogated either by pharmacological or molecular inhibition of connexin 43. In contrast, an extracellular pathway seemed to underlie bystander effects in breast cancer cell lines. These results may impact development of novel therapeutic strategies to selectively eliminate tumors or minimize the size of tissue injury in degenerative or traumatic cell death.

222-Pos Board B22

Effect of Ionic Strength on Bax:TBID Mediated Mitochondrial Outer Membrane Permeabilization (MOMP)

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Fluctuations in ionic strength have been documented in apoptosis. While the exact molecular identity of the channels through which proteins permeate across the outer mitochondrial membrane is unclear, it is fairly certain that the channel is large in size and sustained over time. We sought to determine the effect of salt concentration on Bax and tBid in inducing MOMP. While high salt buffer (60mM) facilitated more release of inter-membrane space (IMS) proteins (Adenylate Kinase and Sulfite Oxidase) with Bax and tBid than low salt (5mM HEPES buffer), low salt conditions induced more sustained, real-time permeabilization, as measured by accessibility of exogenous cytochrome c to complex IV. There was a dichotomy between the kinetics of IMS protein release and real-time permeabilization at high salt conditions, while under low salt conditions, they were congruent. The effect of ionic strength on sustained permeabilization is biphasic, with maxima at 10mM. The sustained permeabilization is inducible by diluting the salt concentration to that of low salt, but irreversible by incorporating high salt conditions. This observation is inconsistent with a dynamic channel model sensitive to ionic strength. It seems likely that under low salt conditions, there is a co-operative interaction between Bax molecules in the membrane and those in solution, but the growth of the channel by membrane-bound Bax molecules itself is independent of ionic strength. Supported by a grant from NSF (MCB-0641208).

223-Pos Board B23

Bax, Bcl-xl Exert their Regulation on Different Sites on the Ceramide Channel

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Ceramide is a sphingolipid that has been shown to play a vital role in the commitment of a cell to apoptosis. There is increasing evidence that ceramide channels may be the pathway through which cytochrome c is released from mitochondria, a critical step in the apoptotic process. Ceramide content increases in mitochondria upon an apoptotic signal and can form stable channels in mitochondrial membranes which are large enough for the passage of proteins. The Bcl-2 family of proteins regulate apoptosis and have pro-apoptotic members, like Bax, and anti-apoptotic members, like Bcl-xL. These proteins have been shown to directly interact with ceramide channels; Bax directly enlarges ceramide channels and Bcl-xL directly disassembles ceramide channels. The molecular site of interaction of Bax and Bcl-xL with ceramide was probed using structural analogs of ceramide which retain channel forming ability. Mitochondrial outer membrane permeability, and thus channel formation, was assessed using the dynamic cytochrome c accessibility assay. The results indicate that Bax was most sensitive to changes in the head group of ceramide while Bcl-xL was most sensitive to changes in tail length. These changes were not simply an effect of kinetics as longer incubations did not yield different results. Furthermore, when we tested known inhibitors of Bcl-xL, 2-methoxyantimycin A3 and ABT-737, the inhibitory effects of Bcl-xL on ceramide could be reversed. The results obtained in this study paint a very novel picture of how proteins may indeed be able to identify and regulate ceramide channels during apoptosis. Supported by a grant from NSF (MCB-0641208).

224-Pos Board B24

Signaling Complexes Transferred by Outer Mitochondrial Membrane Mixing

David Weaver, Veronica Eisner, Xingguo Liu, Peter Varnai, Laszlo Hunyady, Gyorgy Hajnoczky.

The outer surface of the mitochondrion is home to a host of important signaling complexes. While it has been shown that fusion is an important regulator of mitochondrial health through the mixing of matrix contents and mtDNA complementation, little is known about the mixing of membranes, per se or of membrane associated proteins. Here we demonstrate by a PEG cell-fusion assay that two outer membrane associated signaling proteins–the A kinase anchoring protein, AKAP1 and the pro-apoptotic BCL-2 family member, BAD_are efficiently transferred during membrane mixing events. Using photoactivatible GFP fused to the targeting sequence of AKAP1 in combination with matrix targeted DsRed, we found that such events usually lead to the mixing of matrix contents within seconds, but in a fraction of cases the organelles separate without doing so, indicating a potentially distinct function and mechanism for the mixing of the outer membrane and associated proteins.

225-Pos Board B25

Mitochondrial Fusion Dynamics in Adult Rat Skeletal Muscle

Veronica Eisner, Gyorgy Hajnoczky.

Skeletal muscle metabolism and physiology depends on mitochondria function. Impaired mitochondrial function is associated to myopathies and has been suspected to play a role in alcoholic myopathy. A recently recognized determinant of mitochondrial function is mitochondrial fusion-fission dynamics. Whether mitochondria undergo fusion events in adult skeletal muscle is unknown.

We developed an assay to evaluate mitochondria fusion dynamics in adult rat FDB skeletal muscle fibers and satellite cells-derived myotubes expressing mitochondria targeted DsRed (mtDsRed) and photoactivable GFP (mtPAGFP). We used: (1) in vivo electroporated freshly isolated (2 to 24 h) fibers, (2) in vitro adenoviral infected (a) 4 days old fibers and (b) satellite cells-derived skeletal myotubes. Enzymatically isolated fibers co-expressing mtDsRed and mtPAGFP were imaged by confocal microscopy.

Mito-DsRed fluorescence showed intermyofibillar mitochondria arranged in parallel pair rows following the transverse tubules direction. When we tagged the mitochondria in ~5% of total cellular area with two photon photoactivated-GFP, rapid spreading of GFP fluorescence reveled subsets of interconnected mitochondria. Spreading and fusion events occurred mostly in longitudinal direction. Matrix fusion occurred with a frequency of 0.6 ± 0.1 (n=14, mean $\pm$ SE) and 0.3 ± 0.1 events/min/cell (n=12) in fresh and 4 days old fibers, respectively. Skeletal myotubes displayed 6.4 ± 1.5 events/min/cell (n=4). We further evaluated the mitochondrial fusion dynamics in fresh fibers isolated from ethanol and pair-fed (6 month) rats. Fusion events number decreased 33% in fibers coming from ethanol-fed animals. In vitro incubation of fibers with ethanol (80mM, 48h) induced a 97% decrease in the fusion events number (n=8), regarding to control cells. Thus, adult skeletal muscle intermyofibrillar mitochondria undergo fusion that enables mixing of soluble matrix components. Skeletal muscle cells dynamics is dependent on the differentiation stage. Chronic ethanol exposure significantly suppresses fusion dynamics that might contribute to muscle mitochondria and contractile dysfunction.

226-Pos Board B26

Transient Fusion Maintains Mitochondrial Function in Autosomal Dominant Optic Atrophy Associated with Opa1c.984G>A Mutation

Xingguo Liu, Guy Lenaers, György Hajnóczky.

We have identified two classes of fusion events in mammalian cells: complete fusion and transient fusion. In transient fusion, two mitochondria exchanged soluble intermembrane-space and matrix proteins without equilibration of the integral membrane proteins and reseparated preserving the original morphology. Although Opa1, the inner mitochondrial membrane fusion protein is required for both complete and transient fusions, less Opa1 is sufficient to support transient fusion. To understand the specific role of transient fusion in mitochondrial maintenance, we searched for human conditions that display Opa1 loss. Human mutations in Opa1 are associated with Opa1 depletion and cause autosomal dominant optic atrophy (ADOA). Some Opa1 mutations result in mitochondrial fragmentation but the patients' mitochondrial metabolism is well preserved and the clinical symptoms are mild. We reasoned that transient fusion might be retained and support mitochondrial metabolism in these patients. Here, we studied skin fibroblasts derived from a patient who carries the mutation c.984G>A in the GTPase domain of Opa1 and has only weak visual and metabolic impairments, and fibroblasts of two unaffected individuals. In cells bearing Opa1c.984G>A, the two major Opa1 bands were reduced. The majority of the Opa1c.984G>A fibroblasts showed fragmented mitochondria (partial 45%, complete 17%) compared with elongated mitochondria in the controls. In c.984G>A cells with partially fragmented mitochondria, most fusion events were transient, whereas complete fusion dominated in the control cells. Strikingly, the deltapsi, ATP level and mtDNA content were maintained

in Opa1c.984G>A cells. Thus, transient fusions are spared and seem to be sufficient to support mitochondrial metabolism in Opa1c.984G>A fibroblasts, exposing a mechanism that may contribute to the lack of severe clinical symptoms in ADOA associated with some Opa1 mutations.

227-Pos Board B27

Mitochondrial Ca^{2+} Uptake; Regulation by Ca^{2+}, Inhibition by Minocyclin

György Csordás, Tünde Golenár, György Hajnóczky.

Mitochondrial Ca^{2+} uptake is mediated by the low-affinity Ca^{2+} uniporter (MCU) that is controlled allosterically by Ca^{2+}. The Ca^{2+} effect might be mediated by MICU1, a recently identified obligatory component of MCU. Ca^{2+} induces sensitization of MCU but some reports claimed Ca^{2+}-induced desensitization. Here, we compared recovery kinetics of cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_c$) elevations following addition of varying Ca^{2+} doses in suspensions of permeabilized RBL-2H3 cells. The recovery rates (reflecting mitochondrial Ca^{2+} uptake) progressively increased with the Ca^{2+} dose. The $[Ca^{2+}]_c$ recovery steady state was similar after 7.5-50uM $CaCl_2$ pulses, but was reached faster after the larger Ca^{2+} doses. Thus, a pre-exposure to high $[Ca^{2+}]_c$ enhanced the permeability of MCU in a prolonged manner resulting in accelerated mitochondrial Ca^{2+} uptake even at low $[Ca^{2+}]_c$. We are currently investigating the molecular mechanism underlying the effect of Ca^{2+}.

The most specific pharmacological inhibitor of MCU is Ru360 that has limited plasma membrane permeability. Minocyclin, an anti-inflammatory drug has been suggested to exert its cytoprotective and anti-apoptotic effect via interfering with mitochondrial Ca^{2+} uptake, based on studies of isolated mitochondria. However, minocyclin has also been shown to act as a Ca^{2+}-dependent protonophore. We found that minocyclin inhibits the rapid phase of IP_3-induced mitochondrial matrix $[Ca^{2+}]$ response at concentrations of 80-120uM in permeabilized RBL-2H3 cells almost as effectively as Ru360 (80% vs. 92% inhibition, respectively). In minocyclin-pretreated cells, the IP_3-induced Ca^{2+} release caused only slow and small mitochondrial depolarization, while bulk $[Ca^{2+}]_c$ elevation by addition of large amounts of Ca^{2+} elicited almost complete depolarization. These data suggest that the protonophore action of minocyclin requires relatively large $[Ca^{2+}]_c$. Thus, minocyclin seems to be an effective inhibitor of mitochondrial Ca^{2+} uptake sites during physiological $[Ca^{2+}]_c$ signals and exerts its protonophore effect under conditions of large global Ca^{2+} exposure.

228-Pos Board B28

Regional Specialization of Mitochondrial Ca^{2+} Signaling in Cardiac Cells

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Excitation-contraction coupling in cardiac cells is controlled mainly by I_{Ca}-gated release of Ca^{2+} from the sarcoplasmic reticulum (SR), but is also thought to be modulated by mitochondrial Ca^{+} signaling. Using field-stimulated rat ventricular cardiomyocytes, we have previously found that mitochondria that were primed by rapid beating or caffeine-induced SR-Ca^{2+} release subsequently were capable of rapid release of Ca^{2+} when stimulated by shear forces (Belmonte and Morad, 2008, J. Physiol. **586**: 1379). To elucidate this finding we measured mitochondrial Ca^{2+} using Mitycam. The adenoviral Mitycam probe was originally created using the inverse pericam mutant M13-EYFP (V68L/Q69K)(145-238) and EYFP(V168L/Q69K)(1-144)-CaM, fused to the subunit VIII of human cytochrome *c* oxidase mitochondrial-targeting signal (Kettlewell *et al*., 2009, J Mol Cell Cardiol, **46**:891). Short-time cultured adult feline cardiomyocytes were infected with the mitycam adenovirus at MOI of 200 virus particles per cell. After 3-4 days we measured the Mitycam fluorescence under voltage-clamp conditions using whole-cell fluorometry and 2-D confocal imaging. Supporting information was obtained using TMRE to assess mitochondrial depolarization and NCX-currents to assess cytosolic Ca^{2+} transients. The whole-cell Mitycam measurements suggested transfer of Ca^{2+} from the SR to the mitochondria during and following voltage-clamp depolarizations and caffeine-induced SR Ca^{2+} release. In confocal measurements the distribution of Mitycam-fluorescence showed longitudinal streaks with sarcomeric banding resembling the TMRE staining and a characteristic nuclear band (NB) of enhanced fluorescence that typically connected and extended from the two nuclei. Depolarization, caffeine, mechanical stimulation, and FCCP produced changes in Mitycam fluorescence that often resulted in redistribution between NB, general mitochondrial, and subsarcolemmal mitochondrial compartments. Similarly, injection of FCCP caused local decline in TMRE fluorescence (mitochondrial depolarization), but strongly enhanced subsarcolemmal fluorescence. These findings suggest that mitochondria in different regions of cardiac cells play different roles in Ca^{2+} signaling. NIH HL16152.

229-Pos Board B29

Quantification of Mitochondrial Calcium Dynamic Changes During Voltage-Induced Calcium Release in Mammalian Skeletal Muscle

Jianxun Yi, Yan Li, Eduardo Rios, Changling Ma, **Jingsong Zhou**.

Mitochondrial Ca^{2+} uptake regulates mitochondrial metabolism and synthesis of ATP to meet demands of muscle contraction. In a recent study, we found that mitochondrial Ca^{2+} uptake also plays a critical role in modifying rapid Ca^{2+} transients in skeletal muscle of amyotrophic lateral sclerosis transgenic mice (*Zhou 2010*). To better understand how mitochondria are involved in the control of Ca^{2+} transients in healthy and diseased conditions, we need to trace dynamic changes of Ca^{2+} inside mitochondria during contractile activation. *Rudolf* (*2004*) first demonstrated mitochondrial Ca^{2+} uptake in skeletal muscle during contraction using the cameleon YC2. The maximal ratio change, however, was not more than 0.4, and Ca^{2+} uptake was not quantified. We have now used the improved biosensor YC3.6 (*Nagai 2004*) with a dynamic range close to 6 (in *in-situ* calibration) to monitor changes of free $[Ca^{2+}]$ inside mitochondria during voltage clamp-induced Ca^{2+} release (VICR). We targeted YC3.6 to mitochondria by adding a mitochondrial signal sequence at 5' of the cDNA to obtain mt11-YC3.6. One week after FDB muscles of adult mice were transfected by electroporation, enzyme-isolated single FDB fibers expressing mt11-YC3.6 were patch-clamped. Following a depolarizing pulse, the cytosolic Ca^{2+} transient (monitored by x-rhod-1) and the change of Ca^{2+} inside mitochondria were simultaneously recorded in a confocal microscope. We found that the maximal ratio change of mt11-YC3.6 reached 3.25 following a membrane depolarization. The free $[Ca^{2+}]$ inside mitochondria during VICR was calculated using parameters from an *in-situ* calibration. $[Ca^{2+}]_{mito}$ increased to 200 nM during a 10 ms pulse and reached 3 μM during a 800 ms pulse. These results and similar measurements in progress will allow us to evaluate the uptake of Ca^{2+} by mitochondria during single twitches and tetanic contraction. Supported by MDA/NIAMS.

230-Pos Board B30

Isoform-And Species-Dependent Sensitization of the IP3 Receptor by Superoxide Anion

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Multiple interactions have been described between reactive oxygen species and calcium signaling but the underlying mechanisms remain elusive. In human HepG2, rat RBL-2H3, and chicken DT40 cells, we observed cytoplasmic [Ca2+] ([Ca2+]c) spikes and frequency-modulated oscillations evoked by a superoxide anion donor, xanthine (X)+xanthine oxidase (XO), dose-dependently. The [Ca2+]c signal was also observed when extracellular Ca2+ was removed or mitochondria were uncoupled but was eliminated upon ER Ca2+ depletion by thapsigargin, indicating that superoxide stimulated ER Ca2+ mobilization. To test if superoxide affects the IP3 receptor (IP3R), we measured the IP3-induced Ca2+ mobilization in the presence or absence of X+XO in permeabilized cells. X+XO promoted the response to submaximal doses of IP3 but did not change the effect of maximal IP3, indicating sensitization of the IP3R by superoxide. To test the sensitivity of each IP3R isoform to superoxide we used DT40 cells lacking two out of three (DKO) or all IP3R isoforms (TKO). In response to X+XO, DKO expressing either type 1 (DKO1) or type 2 IP3Rs (DKO2) showed a [Ca2+]c signal, whereas DKO expressing type 3 IP3R (DKO3) or the TKO did not show a [Ca2+]c elevation. By contrast, IgM that stimulates IP3 formation, elicited a [Ca2+]c signal in every DKO and caused no [Ca2+]c increase in the TKO. These results indicate that each IP3R isoform could be activated by IP3 but only IP3R1 and IP3R2 are sensitized by superoxide. Further supporting this conclusion, X+XO facilitated the Ca2+ release evoked by submaximal IP3 in permeabilized DKO1 and DKO2 but was not effective in DKO3. Surprisingly, X+XO could also facilitate the effect of low IP3 in TKO transfected with rat IP3R3. We are currently studying if the molecular structure of IP3R3 might explain the isoform and species-dependent sensitization by superoxide.

231-Pos Board B31

Resin-Assisted Capture Methods Show that *S*-Nitrosylation Exerts Cardioprotection During Ischemia/Reperfusion Injury by Directly Reducing Cysteine Oxidation

Mark J. Kohr, Junhui Sun, Angel Aponte, Guanghui Wang, Marjan Gucek, Charles Steenbergen, Elizabeth Murphy.

Redox modifications play an important role in many cellular processes, including cell death. Ischemic preconditioning (IPC) has been shown to involve redox signaling and protein *S*-nitrosylation (SNO) is greatly increased following